



UNIVERSITI PUTRA MALAYSIA

**THERMOSTABILITY OF THE RECOMBINANT
HAEMAGGLUTININ-NEURAMINIDASE
GLYCOPROTEINS OF NEWCASTLE DISEASE VIRUS**

TANG YIK KIONG

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By

TANG YIK KIONG

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirements for the Degree of Master of Science**

July 2003



*Dedicated to,
Yik-Shin, Yik-Jia, Parents, Relatives and
My Friends Graduated from Taiwan...*

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in the fulfilment of the requirements for the degree of Master of Science

THERMOSTABILITY OF THE RECOMBINANT HAEMAGGLUTININ-NEURAMINIDASE GLYCOPROTEINS OF NEWCASTLE DISEASE VIRUS

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July 2003

Chairperson : Prof Datin Khatijah Mohd Yusoff, Ph.D.

Faculty : Science and Environmental Studies

The haemagglutinin-neuraminidase (HN) glycoprotein of Newcastle disease virus (NDV) is of primary importance in inducing virus-neutralizing antibodies against viral infection in chicken and has been used in the development of many vaccines. A variant strain of the vaccine strain *V4QUE* known as *V4UPM(HR)* has been developed as a heat stable vaccine for use in the poultry industry in tropical countries such as Malaysia. This protein may also be involved in maintaining heat stability of some vaccine strains. In this study, the HN gene of the heat stable variant NDV strain *V4UPM(HR)* and its parental strain *V4QUE* were cloned and expressed in the Baculovirus Expression Vector System (*BEVs*) and characterized for their heat stability.

The 1.9 kb HN genes of these strains were amplified by RT-PCR from their genomic RNA and unidirectionally cloned into the baculovirus transfer plasmid, *pCR Bac4.8*. These

recombinant baculovirus plasmids were then co-transfected with linearized baculoviral DNA, *Bac-N-Blue*TM DNA into *Spodoptera frugiperda* (*Sf9*) insect cell line. The recombinant baculoviruses which were generated as *recHNV4UPM(HR)* and *recHNV4QUE*, were purified by plaque assay. The respective recombinant HN glycoproteins (*recHNs*) which were expressed in *Sf9* insect cells showed haemagglutination (HA) and neuraminidase (NA) activities as well as haemagglutination inhibition (HI) and haemadsorption activities in serological assays. The HA and NA activities were also detected on the surface and in the cytoplasm of the infected *Sf9* cells. SDS-PAGE and Western blot analysis of the recombinant baculovirus-infected *Sf9* cell lysates detected protein bands of approximately ~74 kDa, which corresponded to the glycosylated HN protein of the virion. These results indicated that the *recHNs* were not only successfully expressed in the *Sf9* cells but they also appeared to be biologically active and functional.

Based on HA activity, the thermostabilities of *recHNV4QUE* and *recHNV4UPM(HR)* together with *recHNAF2240* on HA activity were studied and compared with those of the NDV strains, *V4QUE*, *V4UPM(HR)* and *AF2240*. The latter was earlier showed to be heat stable at 56°C. The results showed that the heat resistance phenotypes of the recombinant baculoviruses were genetically represented identical to NDV individuals in the property of thermostability. NDV heat resistant strains *AF2240*, *V4UPM(HR)* and recombinant baculoviruses *recHNAF2240*, *recHNV4UPM(HR)* were 50% heat inactivated at ~56°C after 4 hours but the parental NDV strain *V4QUE* and baculovirus strain *recHNV4QUE* remained as the temperature sensitive strains.

In addition, the HN genes of the recombinants were sequenced and analyzed by the secondary and three dimensional structure predictions of the computer programs. The roles of individual amino acid residue(s) of the HN protein in thermostability were discussed. The polar/non-polar side chains of the substituted amino acid residues [R32, V413E, N79K in strain *V4UPM(HR)*; E494K in strain *V4UPM*], where the polar side chain could generate the dipole to form the hydrogen bonding with the aqueous environment and increased the thermostability of the protein. The hydrophobic values and secondary structural arrangements of these substituted/deleted amino acid residues [F151Y, I175M and G431S in strain *V4UPM*; A152T, N276K, I280T, V303T, Q372R and Y504H in strain *AF2240*] and [R32, G221, R305, V413E and S584A in strain *V4UPM(HR)*; H87Q and E494K in strain *V4UPM*] formed the secondary structure of the protein for the protein folding events and the protein packing. The thermostable proteins preferred to form the α -helical/ β -sheet structures rather than the mixture of the β -sheet/random coil or random coil structures alone at the putative HN domain (399-*GAEGR*-403) where was predicted by the secondary structure arrangement. Besides, the 3D structures of the HN protein supported that any amino acid residue substitutions near the putative active sites (HN domain 399-*GAEGR*-403) could have changed the tertiary structure of the HN protein and these tertiary structure/structural arrangements of the HN protein influenced the heat stability of the HN protein.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia untuk memenuhi keperluan Ijazah Master Sains

**KESTABILAN TERMA GLIKOPROTEIN HEMAGLUTININ-NEURAMINIDASE
REKOMBINAN DARI VIRUS PENYAKIT NEWCASTLE**

Oleh

TANG YIK KIONG

Julai 2003

Pengerusi : Prof Datin Khatijah Mohd Yusoff, Ph.D.

Faculti : Sains dan Pengajian Alam Sekitar

Glikoprotein hemagglutinin-neuraminidase (HN) dari virus penyakit Newcastle (NDV) adalah penting dalam merangsangkan antibodi nyah-virus yang berupaya menentang jangkitan virus dalam ayam, maka ia telah banyak digunakan dalam penghasilan vaksin. Satu strain asing dari strain vaksin *V4QUE* yang dikenali sebagai *V4UPM(HR)* telah dikembangkan sebagai vaksin stabil haba untuk kegunaan industri penternakan di negara tropika misalnya Malaysia. Protein in mungkin juga terlibat dalam mengekalkan kestabilan haba untuk sesetengah strain vaksin. Dalam kajian ini, gen HN dari strain NDV asing *V4UPM(HR)* dan strain induknya *V4QUE* telah diklon dan diekspres dalam Sistem Vektor Ekspresi Baculovirus (*BEVs*) dan telah ditentupastikan kestabilan habanya.

Gen HN yang bersaiz 1.9 kb dari strain NDV tersebut telah digandakan dari RNA genomiknya melalui RT-PCR dan seterusnya diklon secara uni-arah ke dalam plasmid pemindah baculovirus, *pCR Bac4.8TM*. Plasmid-plasmid rekombinan baculovirus ini

kemudiannya telah ditranfeksi bersama DNA baculovirus, *Bac-N-Blue*TM yang dilinearkan, ke dalam sel serangga *Spodoptera frugiperda* (*Sf9*). Baculovirus rekombinan yang dinyatakan sebagai *recHNV4UPM(HR)* dan *recHNV4QUE* ini, telah dituliskan melalui asai plak. Glikoprotein HN rekombinan (*recHN*) yang dihasilkan dalam sel serangga *Sf9* ini menunjukkan aktiviti-aktiviti hemaglutinasi (HA) dan neuraminidase (NA), dan juga aktiviti-aktiviti perencatan hemaglutinasi dan hemaserapan dalam asai-asai serologi. Namun aktiviti-aktiviti HA dan NA ini juga dapat dikesan pada permukaan mahupun dalam sitoplasma sel-sel *Sf9* yang terjangkit. Analisis SDS-PAGE dan Western blot terhadap lisat sel *Sf9* yang dijangkiti baculovirus rekombinan telah menunjukkan jalur protein pada lebih kurang 74 kDa, di mana ia bepadanan dengan protein HN yang berglikolasi dari virus. Keputusan ini menandakan *recHNs* bukan sahaja telah berjaya dihasilkan dalam sel-sel *Sf9*, bahkan ia menampilkan keaktifan dan berfungsi biologinya.

Berdasarkan aktiviti HA, kestabilan terma *recHNV4QUE* dan *recHNV4UPM(HR)* bersama dengan *recHNAF2240* telah dikaji dan dibandingkan dengan strain-strain NDV lain, misalnya *V4QUE*, *V4UPM(HR)* dan *AF2240*. Namun begitu, strain-strain yang kemudian itu menunjukkan bahawa ia mengekalkan kestabilan haba pada suhu ~56°C terlebih dahulu. Keputusan-keputusan menunjukkan fenotip rintangan haba bagi baculovirus-baculovirus rekombinan adalah bersamaan secara genetik dengan individu-individu NDV dari segi sifat kestabilan terma. Strain-Strain rintang haba seperti *AF2240* dan *V4UPM(HR)* serta baculovirus rekombinan *recHNAF2240* dan *recHNV4UPM(HR)* dinyahaktifkan sebanyak 50% pada ~56°C selepas 4 jam. Namun begitu, strain induk NDV

V4QUE dan baculovirus rekombinan strain *recHNV4QUE* tetap merupakan strain sensitif haba.

Gen-gen HN rekombinan ini turut diujuk dan protein-protein telah dianalisis melalui ramalan struktur sekunder dan tiga dimensi. Peranan-peranan jujukan asid amino individu pada protein HN dalam kestabilan termanya juga dibincangkan. Rantai sisi berkutub/tak berkutub pada asid amino yang digantikan residues [R32, V413E, N79K di strain *V4UPM(HR)*; E494K di strain *V4UPM*], di mana rantai sisi berkutub dapat mewujudkan dwikutub untuk pembentukan ikatan hidrogen dengan persekitaran akueous dan peningkatan kestabilan terma sesuatu protein. Nilai-nilai hidrofobik dan penyusunan struktur sekundernya pada jujukan asid amino terganti [F151Y, I175M, G431S di strain *V4UPM*; A152T, N276K, I280T, V303T, Q372R, Y504H di strain *AF2240*] dan [R32, G221, R305, V413E, S584A di strain *V4UPM(HR)*; H87Q, E494K di strain *V4UPM*] membentuk struktur sekunder protein bagi pelipatan dan penyusunannya. Protein-protein yang stabil haba lebih cenderung untuk membentuk struktur heliks- α /kepingan- β daripada campuran kepingan- β /lingkaran rawak atau struktur lingkaran rawak sahaja pada domain HN anggapan (399-*GAEGR*-403) seperti yang diramalkan menerusi penyusunan struktur sekundernya. Selain itu, struktur 3D protein HN menyokong bahawa sebarang pergantian jujukan asid amino berhampiran dengan tapak domain aktif anggapan (domain HN 399-*GAEGR*-403) mungkin akan mengubah struktur tertiar protein HN dan struktur tertiar/penyusunan struktur protein HN. Ia seterusnya mempengaruhi kestabilan haba pada protein HN.

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I certify that a Supervisory Committee met on 4th August 2003 to conduct the final examination of Tang Yik Kiong on his Master of Science thesis entitled “Thermostability of The Recombinant Haemagglutinin-neuraminidase Glycoproteins of Newcastle Disease Virus” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Supervisory Committee are as follows:

AINI IDERIS, Ph.D

Professor
Dean, School of Graduate Studies
Universiti Putra Malaysia
(Chairman)

DATIN KHATIJAH MOHD YUSOFF, Ph.D.

Professor
Head, Department of Biochemistry and Microbiology
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

ABDUL MANAF ALI, Ph.D.

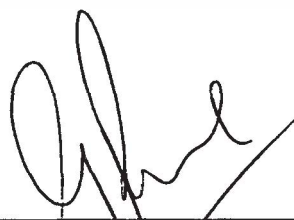
Professor
Institute of BioScience
Universiti Putra Malaysia
(Member)

TEY BENG TI, Ph.D.

Lecturer
Department of Chemical Engineering
Faculty of Engineering
Universiti Putra Malaysia
(Member)

ALAN ONG HAN KIAT, Ph.D.

Lecturer
Division of Chemistry and Biology
School of Art and Science
Tunku Abdul Rahman College
(Member)



GULAM RUSUL RAHMAT ALI, Ph.D.

Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 26 SEP 2003

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are as follows;

DATIN KHATIJAH MOHD YUSOFF, Ph.D.

Professor
Head, Department of Biochemistry and Microbiology
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Chairman)

ABDUL MANAF ALI, Ph.D.

Professor
Institute of BioScience
Universiti Putra Malaysia
(Member)

TEY BENG TI, Ph.D.

Lecturer
Department of Chemical Engineering
Faculty of Engineering
Universiti Putra Malaysia
(Member)

ALAN ONG HAN KIAT, Ph.D.

Lecturer
Division of Chemistry and Biology
School of Arts and Science
Tunku Abdul Rahman College
(Member)



AINI IDERIS, Ph.D.
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia.

Date: **14** NOV 2003

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.


TANG YIK KIONG
Date: 23 SEP 2003

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL	xi
DECLARATION	xiii
LIST OF FIGURES	xviii
LIST OF TABLES	xx
LIST OF ABBREVIATIONS	xxi
LIST OF AMINO ACIDS AND ABBREVIATIONS	xxiv
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	4
2.1 Newcastle Disease	4
2.1.1 Genome Organization and Virion Properties of NDV	4
2.2 Control of Newcastle Disease in Malaysia	5
2.3 Thermostability of Newcastle Disease Vaccine	6
2.4 Thermostability of Proteins	9
2.4.1 The Effects of Temperature on Protein Structure	9
2.4.2 Factors that Enhance Protein Thermostability	10
2.5 The Haemagglutinin-neuraminidase (HN) Gene of Newcastle Disease Virus	12
2.5.1 The Biological and Molecular Characteristic in Temperature Sensitive Mutants of the Newcastle Disease Virus	13
2.5.2 The Crystal Structure of the Multifunctional HN Glycoprotein	14
2.6 The Role of Individual Amino Acid Residues in the Formation of the Stable, Functional HN Glycoprotein of NDV	16
2.6.1 The Role of the Cysteine Residues and Disulfide Bond Arrangement in HN Gene	16
2.6.2 The Role of the Oligosaccharide Chains in HN Gene	18
2.6.3 Amino Acid(s) Changes of the HN Gene	19
2.7 Baculovirus Expression Vector System (<i>BEVs</i>)	21
2.7.1 Biology and Classification of Baculovirus	21
2.7.2 Baculovirus Virion and Genome Structure	21
2.7.3 Principles of <i>BEVs</i>	24
2.7.4 Insect Cell <i>Spodoptera frugiperda</i> (<i>Sf9</i>) Culture and Characteristics of Insect Cell Lines	25
2.7.5 Insect Cell Culture Medium	26
2.7.6 Allelic Replacement of Homologous Recombinant Techniques in <i>BEVs</i>	27



2 7 7	Advantages of the <i>BEVs</i>	28
2 8	Computational Biology and Bioinformatic	31
3	MATERIALS AND METHODS	35
3 1	Chemicals, Reagents, Solvents and Tissue Culture Materials	35
3 2	Viruses, Bacteria and Insect Cell Line	35
3 3	Vectors, Viral DNA, Plasmids and Primers	37
3 4	Buffers, Antibiotics Solution and Media	37
3 5	Nucleic Acid Extraction	41
3 5 1	NDV Viral RNA Extraction	41
3 5 2	Baculoviral DNA Extraction from the Infected <i>Sf9</i> Cell	41
3 6	RT-PCR Procedures	43
3 6 1	RT-PCR Reaction	43
3 6 2	Purification of RT-PCR Product	44
3 7	Cloning, Screening and Verification of Putative Recombinant Clones	45
3 7 1	Ligation of RT-PCR Product into Baculovirus Transfer Vector	45
3 7 2	Preparation of Competent Cells	45
3 7 3	Introduction of the HN Gene into Competent Cells	45
3 7 4	Small Scale Plasmid DNA Extraction	46
3 7 5	Restriction Enzyme (RE) Analysis of the Recombinant Baculovirus Transfer Plasmid	47
3 7 6	PCR Verification of the HN Gene	47
3 7 7	DNA Sequencing	48
3 7 8	RNA Sequencing	48
3 8	Tissue Culture Techniques of the Insect Cell	49
3 8 1	General Handling Techniques	49
3 8 2	Insect Cell Culture Medium	50
3 8 3	Heat-Inactivation of the Fetal Bovine Serum	50
3 8 4	Monolayer Cell Culture and Passaging (Subculturing) Cell Culture	51
3 8 5	Cryopreservation and Storage of Insect Cell Line	51
3 9	Transfection Procedures	52
3 9 1	Co-transfection of the Recombinant Transfer Vector and the Linearized Baculoviral DNA	52
3 9 2	Post-transfection Procedure	53
3 10	Viral Plaque Assay and Identification of Putative Recombinant Baculovirus	53
3 10 1	Preparation of <i>Sf9</i> Insect Cells and Dilution of Recombinant Viruses	53
3 10 2	Viral Plaque Assay	54
3 10 3	Visual Screening for Blue Plaques	55
3 10 4	PCR Verification	55
3 10 5	Partial DNA Sequencing at End Termini of the Recombinant Baculoviral DNA	56

3.11	Conformation of the Expressed HN Protein in Baculovirus Expression System	57
3.11.1	Haemagglutinin Activity (HA)	57
3.11.2	Neuraminidase Activity (NA)	57
3.11.3	Haemagglutination Inhibition (HI)	58
3.11.4	Haemadsorption Assay	58
3.11.5	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of the Baculovirus Infected <i>Sf9</i> Cells Lysis	59
3.11.6	Western Blot Analysis of the Recombinant HN Glycoprotein	60
3.12	Thermostability Studies of the Recombinant HN Protein	61
3.12.1	Thermostability Assay of the Haemagglutinin Activity	61
3.12.2	Stability Studies of the Haemagglutinin Activity	61
4	RESULTS AND DISCUSSION	62
4.1	Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) and Polymerase Chain Reaction (PCR) of NDV Nucleic Acid	62
4.1.1	Amplification of HN Gene by RT-PCR	62
4.2	Cloning of the HN Gene into a Cloning and Shuttle Vector	63
4.2.1	Cloning of the HN Gene into a Cloning Vector, <i>pCR2.1</i>	63
4.2.2	Subcloning of the HN Gene into a Shuttle Vector, <i>pCR Bac4.8</i>	65
4.2.3	Restriction Enzymes (RE) Analysis and PCR Verification of the Recombinant Baculovirus Transfer Plasmids	67
4.2.4	Partial End Termini DNA Sequencing of the Putative Recombinant Plasmids	72
4.3	Morphology of the <i>Sf9</i> Cells and the Infected <i>Sf9</i> Cells Culture	72
4.3.1	General Morphology of the <i>Sf9</i> Cells	72
4.3.2	Morphology of the Baculoviral Infected <i>Sf9</i> Cells	72
4.4	Co-transfection of the Recombinant Baculovirus Plasmid with Linearized Baculoviral DNA into the <i>Sf9</i> Insect Cells	73
4.4.1	Confirmation of the Transfection Reaction	73
4.4.2	Identification of the Putative Recombinant Baculovirus by Plaque Assay	77
4.4.3	Identification of the Putative Recombinant Baculovirus by PCR Procedure	79
4.4.4	Partial DNA Sequencing at the End Termini of the Recombin Viral DNA	79
4.5	Expression of the HN Glycoprotein in the <i>Sf9</i> Cells	82
4.5.1	Serological Assay and Haemagglutination Inhibition Test	82
4.5.2	SDS-PAGE and Western Blot Analysis of the Recombinant HN Glycoprotein	86
4.6	Thermostability Assay of NDV Strains and the Recombinant Baculoviruses on HA Activities	92
4.6.1	Thermostability Assay of NDV Strains and the Recombinant Baculoviruses	92
4.6.2	Stability Response of the HA Activity at 56°C Heat Stress	93

4.7	Thermostability of the HN Polypeptide	96
4.7.1	Single Amino Acid Residues Change in the HN Polypeptide of NDV Strain <i>V4UPM(HR)</i>	101
4.7.2	Single Amino Acid Residues Change in the HN Polypeptide of NDV Strain <i>V4UPM</i>	107
4.7.3	Single Amino Acid Residues Change in the HN Polypeptide of NDV Strain <i>AF2240</i>	110
4.7.4	Glycosylation Sites and Cysteine Residues of the HN Polypeptide of NDV Strains	115
4.7.5	Identification of a Putative Heat Stable Domain of the HN Polypeptide	116
5	SUMMARY AND CONCLUSION	123
	REFERENCES	127
	APPENDICES	142
	VITA	159

LIST OF FIGURES

Figure		Page
2 1	Schematic Diagram of Various Forms of Baculovirus	22
2 2	The Infection Cycle of the Baculovirus BV and OV in Insect Cells	23
2 3	Illustration of Allelic Replacement Reaction in <i>BEVs</i>	29
4 1	Amplified HN Genes from NDV Strains <i>AF2240</i> , <i>V4QUE</i> , <i>V4UPM(HR)</i>	64
4 2(a)	Screening of the Putative Recombinant Clones by Single Digested with <i>HindIII</i>	66
4 2(b)	Screening of the Putative Recombinant Clones by Double Digested with <i>BamHI</i> and <i>HindIII</i>	66
4 3(a)	Screening of the Putative Recombinant Baculovirus Transfer Plasmid by Double Digested with <i>HindIII</i> and <i>BamHI</i>	68
4 3(b)	Restriction Enzyme Analysis of the Putative Recombinant Baculovirus Transfer Plasmids	69
4 4	PCR Verification of the Putative Recombinant Plasmids	70
4 5	The Recombinant Baculovirus Transfer Plasmid	71
4 6	Partial DNA Sequencing at the End Termini of the Recombinant Baculovirus Transfer Plasmid	74
4 7	<i>Sf9</i> Insect Cell Line	75
4 8	Signs of the Baculoviral Infection	76
4 9	Schematic Diagram of Blue Plaque Formation of the Recombinant Baculovirus	78
4 10	PCR Verification of the Recombinant Baculoviruses	80
4 11	Partial DNA Sequencing at the End Termini of the Recombinant Viral DNA	81
4 12	The Hemagglutinin Activity (HA) of the Recombinant Baculoviruses and Wild Type Baculovirus	83
4 13	The HA Activity of Recombinant Baculoviruses	84
4 14	Haemadsorption Assay of the Recombinant Baculovirus Infected <i>Sf9</i> Cell Culture	85
4 15	The NA Assay was also Done on the Infected Cells Lysates by Using the Quantitative Estimation of <i>N</i> -acetylneuraminic Acid	87
4 16	The Haemagglutination Inhibition Test of Recombinant Baculoviruses	88
4 17	Pattern of Fractionated Protein in 10% SDS-PAGE	90
4 18	Western Blot Analysis of Infected Cell Lysates by Using NDV Antisera Polyclonal Antibodies	91
4 19	Thermostability Assay on the Haemagglutinin Activity (HA) of NDV Strains <i>V4QUE</i> , <i>V4UPM(HR)</i> , <i>AF2240</i> and Recombinant Baculovirus Strains <i>recHNV4QUE</i> , <i>recHNV4UPM(HR)</i> , <i>recHNAF2240</i> . HA Activity Remaining vs Temperature are Plotted	94
4 20	Stability Response of the Haemagglutinin Activity at 56°C Heat Stress HA Activity Remaining vs Hours of Heat Stress are Plotted	95
4 21	CLUSTAL W (1.81) Multiple Sequence Alignment of the HN Protein of NDV Strains <i>V4QUE</i> , <i>V4UPM(HR)</i> , <i>V4UPM</i> and <i>AF2240</i>	97



4.22	: Multiple Sequence Alignment of the HN Polypeptide. Secondary Structure of HN Proteins; α -helix, β -sheet and Random Coil Structure were Predicted	104
4.23	: The Tertiary Structure of the HN Protein	121
4.24	: The Tertiary Structure of the HN Protein Represented in a Ribbon at this Domain	122

LIST OF TABLES

Table	Page
3 1 List of Baculovirus and Recombinant Virus, NDV, Bacteria and Insect Cell	36
3 2 List of Vectors, Linearized Baculoviral DNA and Plasmids	38
3 3 List of Primers	39
3 4 List of Buffers, Antibiotics Solution and Media	40
4 1 Matrix of Amino Acid Residues between Thermophilic and Mesophilic Protein Sequence	99
4 2 Favorable Amino Acid Replacements from Mesophilic to Thermophilic Proteins	100
4 3 Nucleotide Changes, Deduces and Amino Acid Substitution in Heat Stability NDV Strain <i>V4UPM(HR)</i> were Compared to its Published Parental (Heat Sensitive) Strain <i>V4QUE</i>	102
4 4 Nucleotide Changes, Deduces and Amino Acid Substitution in Heat Stability NDV Strain <i>V4UPM</i> were Compared to its Published Parental (Heat Sensitive) Strain <i>V4QUE</i>	108
4 5 Nucleotide Changes, Deduces and Amino Acid Substitution in Heat Stability NDV Strain <i>AF2240</i> were Compared to its Published Parental (Heat Sensitive) Strain <i>V4QUE</i>	111
4 6 List of Amino Acid Residues Change in NDV Strains <i>V4UPM(HR)</i> , <i>V4UPM</i> and <i>AF2240</i> that May Contributed towards the Thermostability of HN Polypeptide	114



LIST OF ABBREVIATIONS

A

A	Ampere
<i>AcMNPV</i>	Autographa Californica Multiple Nuclear Polyhedrosis Virus
<i>Amp</i>	Ampicillin
α	Alpha

B

β	Beta
BCP	1-bromo-3-chloropropane
<i>BEVs</i>	Baculovirus Expression Vector system
<i>BmNPV</i>	<i>Bombyx mori</i> (Silkworm) Nuclear Polyhedrosis Virus
BV	Budded Virus

C

cDNA	Complementary Deoxyribonucleic Acid
CRBC	Chicken Red Blood Cells
CO ₂	Carbon Dioxide

D

ddNTP	Dideoxynucleotide Triphosphates
DEPC	Diethylpyrocarbonate
dH ₂ O	Distilled Water
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate

E

ECV	Extracellular Virus
EDTA	Ethylenediaminetetraacetic Acid Disodium Salt
<i>E. coli</i>	<i>Escherichia coli</i>

F

F	Fusion Protein
Fig.	Figure
FBS	Fetal Bovine Serum

G

<i>Gal</i>	Glyco
g	Gram
xg	x Gravity

H

h	Hour
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HA	Haemagglutinin Activity
HAU	Haemagglutinin Activity Unit
HI	Haemagglutination Inhibition
HN	Haemagglutinin-neuraminidase Protein
HR	Heat Resistance
I	
ICTV	The International Committee in the Taxonomy of Viruses
K	
KCl ₂	Potassium chloride
Kb	Kilobase
kDa	Kilodalton
L	
l	liter
ln	Base 2 Logarithms
L	Polymerase Protein
M	
M	Matrix Protein
m	Milli
ml	Milli Liter
mM	Milli Molar
MCS	Multiple Cloning Sites
min	Minute
M	Molar
<i>Mr</i>	Molecular Weight
N	
NANA	<i>N</i> -aceylneuraminic Acid
NA	Neuraminidase Activity
ND	Newcastle Disease
NDV	Newcastle Disease Virus
n	nino
NOVs	Non-occluded Virus
NP	Nucleoprotein
O	
ooc ⁻	Absence of Occlusion Bodies
ooc ⁺	Presence of Occlusion Bodies
ORF	Open Reading Frame
OV	Occlusion Virus
P	
PBS	Phosphate Buffer Saline

PCR	Polymerase Chain Reaction
PIBs	Polyhedral Inclusion Bodies
<i>polh</i>	Polyhedrin Genes of Baculovirus
P	Phosphoprotein
p	pico
PVDF	Polyvinylidene Difluoride Membranes
R	
RBC	Red Blood Cells
<i>rec</i>	Recombinant
<i>recHN</i>	Recombinant Haemagglutinin-Neuraminidase Glycoprotein
RE	Restriction Enzymes Analysis
RNA	Ribonucleic Acid
rpm	Round per Minute
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RT	Room Temperature
S	
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
sec	Second
<i>Sf-900II</i> SFM	<i>Sf-900II</i> Serum Free Medium
<i>Sf9</i>	<i>Spodoptera frugiperda</i>
SFM	Serum Free Medium
T	
<i>Taq</i>	<i>Thermus Aquaticus</i>
TAE	Tris-acetate EDTA Buffer
TBE	Tris-boric EDTA Buffer
TE	Tris-base Buffer
T _m	Melting Temperature
T _n	<i>Trichoplusia ni</i>
U	
μ	micron
UV	Ultra Violet
U	Unit
UPM	Universiti Putra Malaysia
V	
v	Volume
V	Volt
W	
<i>wt</i>	Wild Type
w	Weight

LIST OF AMINO ACIDS AND ABBREVIATIONS

A	Alanine (Ala)
C	Cysteine (Cys)
D	Aspartic Acid (Asp)
E	Glutamic Acid (Glu)
F	Phenylalanine (Phe)
G	Glycine (Gly)
H	Histidine (His)
I	Isoleucine (Ile)
K	Lysine (Lys)
L	Leucine (Leu)
M	Methionine (Met)
N	Asparagine (Asn)
P	Proline (Pro)
Q	Glutamine (Gln)
R	Arginine (Arg)
S	Serine (Ser)
T	Threonine (Thr)
V	Valine (Val)
W	Tryptophan (Trp)
Y	Tyrosine (Tyr)